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PLAQUE SIZE AND VIRULENCE OF ATTENUATED VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS AFTER PASSAGE IN VARIOUS HOSTS

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Hearn, H. J. (Exp. Aerobiol. Div., Ft. Detrick, Md. 21701), and P. Jameson. Plaque size and virulence of attenuated Venezuelan equine encephalomyelitis virus after passage in various hosts. *Amer J Epidem* 94: 56–61, 1971.—The virulence and plaque properties of a new, small plaque attenuated 20t strain of Venezuelan equine encephalomyelitis (VEE) virus were characterized and compared with those of the moderately attenuated small plaque 9t and the virulent, large plaque parent egg seed strains in a variety of hosts. A large plaque, attenuated viral type was isolated from the small plaque 20t strain by passage in embryonated eggs and a small plaque, virulent strain was isolated by passage in mice. Thus, while plaque size and virulence were shown not to be irrevocably linked for VEE virus, the data provide evidence that deliberate selection and passage in specific laboratory host systems can produce stable viral types whose plaque size and virulence characteristics are correlated.

arbovirus; encephalomyelitis, equine; virulence

INTRODUCTION

An attenuated Venezuelan equine encephalomyelitis (VEE) virus strain, 20t, that possesses a high level of plaque homogeneity and a level of virulence less than that of another attenuated (9t) strain has been described (1, 2). The objective of this work was to obtain additional information on the characteristics of the 20t virus to make it useful as a model in studies designed to evaluate environmental circumstances that promote the selection and perpetuation of attenuated viral populations. More specifically, this report compares virulence of the 20t strain with that of the 9t and nonattenuated parent egg seed (PES) strains and describes two isolates of 20t virus that differ with regard to the size of plaques formed and virulence after passage in embryonated eggs and mice.

MATERIALS AND METHODS

Virus strains. PES strain of VEE virus, Trinidad strain, consisted of a 10 per cent (w/v) suspension of infected chicken embryos at the thirteenth passage level (2). The attenuated 9t and 20t strains of VEE virus were L cell preparations that have been described in detail elsewhere (1, 2).

Preparation of infected tissues. Serial passages of virus in chicken eggs were made with infected embryos that were homogenized in a Waring Blendor with sterile Bacto heart infusion broth to make a 10 per cent (w/v) suspension. Brain and spleen tissues were obtained from infected suckling and young adult mice when they displayed

Abbreviations: ELD_{50}, egg median lethal dose; $MICID_{50}$, mouse median infectivity dose, injected intracerebrally; $MICLD_{50}$, mouse median lethal dose, injected intracerebrally; $MIPID_{50}$, mouse median infectivity dose, injected intraperitoneally; $MIPLD_{50}$, mouse median lethal dose, injected intraperitoneally; PES, parent egg seed; VEE, Venezuelan equine encephalomyelitis.

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clinical signs of disease. In some cases, suckling mouse tissues were removed shortly after death. Each sample to be titrated consisted of tissues that were removed aseptically from three animals, pooled, ground in a TenBroeck grinder and then diluted with sufficient Bacto heart infusion broth to make a 10 per cent suspension. Samples were placed in glass ampoules and stored frozen until titrations for virus were performed.

Cell cultures for plaque assay. For plaque assays, 5 ml of L cells at a concentration of about one million per milliliter in medium 199 supplemented with 10 per cent horse serum (199-HS_{10}), were placed in 60-mm plastic Petri plates. The cells were maintained as monolayers in 199-HS_{10} containing 100 units of penicillin and 0.1 mg of streptomycin per milliliter. The monolayers were incubated for approximately 24 hours at 35 C in a 5 per cent carbon dioxide atmosphere prior to inoculation of virus.

Plaque assay. Monolayers of L cells, from which the supernatant medium had been removed by aspiration, were inoculated with 0.1 ml of virus diluted in Bacto heart infusion broth pH 7.5. After a 15- to 30-minute interval to allow for viral attachment, the monolayers were overlayed with 5 ml of glucose-yeastolate medium containing agar (3). Infected cultures were incubated in an inverted position. On the third day after inoculation, a second glucose-yeastolate-agar medium without horse serum containing 0.006 per cent neutral red was added; plaques were counted the following day.

Virus titrations in mice and eggs. Median lethal dose ($MICLD_{50}$ or $MIPLD_{50}$) titers were determined by injection of 12- to 14-gm Swiss-Webster mice intracerebrally (ic) or intraperitoneally (ip). Immune responses of mice injected with attenuated virus were determined by resistance to a subsequent challenge with a multiple lethal dose ($10^{3.5}$ to $10^{4.5}$ $MICLD_{50}$) of the lethal PES strain of VEE virus 18 to 21 days after the primary injection. Median infectivity ($MICID_{50}$ or $MIPID_{50}$) titers were calculated from the ratios of survivors to the number challenged.

Egg median lethal doses (ELD_{50}) of VEE virus were obtained by injection into the amniotic cavity of 14-day embryonated eggs and by injection of seven-day eggs by the allantoic route. Embryo deaths prior to 18 hours postinoculation were considered nonspecific. Median doses were calculated by the method of Spearman-Karber (4). All titers are expressed as viral units per milliliter.

TABLE 1

Comparison of lethality, infectivity, and plaque titers of virulent parent egg seed and attenuated (9t and 20t) strains of VEE virus

Host and method of expressing assay titer*	Titer values (log_{10}/ml) of viral strains		
	Parent egg seed	9t	20t
Mouse (12- to 14-gm)			
$ICLD_{50}$	9.8	7.8	<1.5†
$ICID_{50}$	9.8	8.2	8.8
$IPLD_{50}$	9.0	<0.6†	<0.6
$IPID_{50}$	9.3	7.8	8.1
Suckling mouse			
$ICLD_{50}$	10.5	9.2	9.5
$ICID_{50}$	10.5	NT‡	9.5
$IPLD_{50}$	10.3	8.6	8.9
$IPID_{50}$	10.3	8.6	9.6
Hamsters (35- to 40-gm)			
$IPLD_{50}$	9.4	8.6	<2.7
$IPID_{50}$	9.6	8.8	8.1
Egg			
(14-day) amniotic LD_{50}	8.5	Sporadic§	<1.0†
(7-day) allantoic LD_{50}	7.4	7.7	8.4
L cell plaque-forming units	9.0	8.6 (0.5–1 mm)	8.8 (0.5–1 mm)

* $ICLD_{50}$, median lethal dose, injected intracerebrally; $ICID_{50}$, median infectivity dose, injected intracerebrally; $IPLD_{50}$, median lethal dose, injected intraperitoneally; $IPID_{50}$, median infectivity dose, injected intraperitoneally.

† No lethality, using dilutions 10^0 through 10^{-6}.

‡ Not titrated.

§ Some virus-specific egg lethality throughout range of dilutions but not of sufficient incidence to allow the computation of an LD_{50} value.

RESULTS

Comparative infectivity and lethality of virulent PES and attenuated 9t and 20t strains in various hosts. As shown in table 1, the 20t strain uniformly was not lethal but infective (immunogenic) for 12- to 14-gm mice by either the ic or ip route. This

TABLE 2

Characteristics of attenuated 20t virus populations after serial passage in embryonated eggs

Virus preparation* (passages)	Titer values (log_{10}/ml) of viral strains							
	ELD_{50}†		pfu‡		MLD_{50}§		MID_{50}¶	
	7-day	14-day	Large	Small	ic	ip	ic	ip
20t	8.2	<1	<2	8.7	<1.5	<0.6	8.8	8.1
E_2	5.3	1.5	3.8	6.0	<2.5	<1.6	6.0	5.3
E_4	6.5	5.5	7.1	6.5	<3.5	<2.6	7.1	6.1

* L cell preparation of 20t virus passed up to four times (E_4) by allantoic route in 7-day embryonated eggs using 10^{-4} dilution of inoculum for successive passages.

† Median lethal dose in embryonated eggs: 7-day injected by allantoic route or 14-day injected by amniotic route.

‡ Large (2–4 mm) or small (0.5–1 mm) plaque-forming units.

§ Median lethal dose in 12- to 14-gm mice injected either intracerebrally (ic) or intraperitoneally (ip).

¶ Median infective (immunizing) dose in 12- to 14-gm mice injected either ic or ip.

contrasted markedly with the PES strain which was highly lethal for mice (titers of $10^{9.8}$ $MICLD_{50}$ and $10^{9.0}$ $MIPLD_{50}$). The similarity between the LD_{50} and ID_{50} values for this strain shows that infectivity invariably resulted in fatality. The distinction between the attenuated 20t and 9t strains was less pronounced, but not equivocal, since the latter strain was lethal ($10^{7.6}$ $MICLD_{50}$) when injected intracerebrally but not intraperitoneally.

Infectivity titers of both attenuated strains in animals were similar to the plaque titers in L cells as shown in table 1. All three strains showed a high level of lethality for suckling mice.

Tests in hamsters disclosed a distinct difference between 20t virus and the other two (PES and 9t) strains. Strain 20t was the only one of the three that was not lethal, but it remained highly immunogenic for these animals.

In embryonated eggs, 20t virus failed to produce lethality in 14-day eggs but was highly lethal ($10^{8.4}$ ELD_{50}) in seven-day eggs. This is in contrast to the PES strain, which showed high titers ($10^{8.5}$ ELD_{50}) in 14-day eggs, yet was approximately one-tenth as lethal for seven-day eggs. The 9t virus showed some evidence of viral lethality in 14-day eggs, but this was always sporadic and did not conform to a typical dilution-dependent response within a titration, thus preventing computation of LD_{50} values.

Passage of the 20t strain in embryonated eggs. As mentioned above, in contrast to the virulent PES strain, the attenuated 20t strain was not lethal for 14-day eggs (table 1). When the 20t strain was serially passed in seven-day eggs, a virus population that was lethal for 14-day eggs appeared (table 2). By the fourth passage, the virus recovered after injection of the seven-day embryos was almost as lethal for 14-day eggs as for seven-day eggs and substantial titers of both large and small plaque-forming virus were found in suspensions of the embryo homogenates. This virus population (E_4) was of interest because it was not lethal for mice injected either intracerebrally or intraperitoneally, even though it contained substantial quantities ($10^{7.1}$ plaque-forming units (pfu)/ml) of large plaque virus.

The PES virus, which is highly lethal for mice, characteristically forms predominantly large plaques in L cells.

To verify the apparent emergence of a new attenuated large plaque type, virus was isolated from large plaques produced by the E_4 population and given four additional serial passages in L cells. Assays were performed in mice and by plaque production at each passage level (not shown in table 2). By the time the second passage was completed, the virus produced plaque titers of $10^{8.3}$ to $10^{8.7}$ pfu/ml; with the exception of an occasional 1.5- or 2-mm plaque, all of the plaques were large (>3 mm) and the virus was not lethal for mice by either the ic or ip route. Mice injected with this virus resisted a multiple lethal challenge with the PES strain.

Passage of the 20t strain in suckling and young adult mice. As previously indicated in table 1, the small plaque-forming 20t strain was uniformly lethal through a wide range of doses for suckling mice. Prelimi-

nary tests with virus recovered from these animals revealed that concentrations of large plaque virus ($10^{2.6}$ to $10^{5.3}$ pfu/ml) were recovered from one-half of the brain samples and from all of the spleen-liver samples after intracerebral injection. After intraperitoneal injection, approximately one-third of the suckling mouse brain and spleen-liver homogenates showed large plaque virus. The incidence of recovery of this large plaque type could not be correlated with the dose of virus that the mice received.

The lethality of low doses of the small plaque 20t virus for suckling mice coupled with the repeated recovery of large plaque virus in spleen-liver tissues after ic injection, but not necessarily after ip injection, indicated that suckling mice readily selected and/or supported back-mutation and replication of the large plaque virus. The fact that lethality could occur in the absence of large plaque virus, however, also suggested that replication of this plaque type may not necessarily be required to induce a lethal infection in this host. This, in turn, gave rise to the supposition that the mice produced VEE virus whose plaque size remained small but whose virulence became increased. The character of the viral changes that took place during replication of the 20t strain in brain and visceral tissues of suckling mice was examined further in a subsequent series of tests. Results showed that virulence and plaque-forming characteristics of the viral progeny varied depending upon the route of administration of the 20t inoculum and the tissue from which the virus was recovered.

As examples, tests on 20 pools of tissue homogenates obtained after injection of the 20t virus in suckling mice disclosed the following. 1) Brain tissue from moribund or dead suckling mice contained $10^{7.8}$ to $10^{7.8}$ pfu of small plaque virus that was uniformly lethal for young adult mice. In contrast, spleen-liver homogenates from the suckling mice showed the same small plaque titers as the brain tissue but the virus remained nonlethal for young adult mice. 2) Brain tissue did not routinely contain virus that was lethal for 14-day eggs, whereas small but detectable titers of $10^{2.2}$ to $10^{4.2}$ ELD_{50}/ml were obtained with spleen-liver samples. 3) Large plaque virus (titers of $10^{3.0}$ to $10^{4.0}$) was recovered from both brain and spleen-liver tissues of mice that were injected intracerebrally but only sporadically from those that were injected intraperitoneally.

On the basis of these results, it was tentatively concluded that suckling mice selected and supported some growth of large plaque virus, provided that the inoculum was administered directly into the brain, but that the brain was most actively engaged in the selection and proliferation of a small plaque virulent type that was lethal for young adult mice. In contrast to brain, spleen-liver tissues appeared most active in producing small plaque virus that was not lethal for young adult mice but that was lethal to some extent for 14-day eggs. The production of large plaque virus was irrelevant to either egg lethality or young adult mouse lethality and, as shown below, this capability became lost upon further passage in young adult mice.

In a final test, two suckling mouse brain samples were selected for further study. One of these had been obtained after the ip injection of 20t virus in suckling mice, and shown to contain a titer of $10^{6.7}$ LD_{50} in young adult mice, a small plaque titer of $10^{7.5}$ and a large plaque titer of $10^{4.0}$ pfu/ml. The other brain sample was obtained from suckling mice after an ic injection; it displayed approximately the same lethality for young adult mice, the same small plaque titer, but no detectable large plaque titer.

Despite the dissimilarity in plaque size distribution between the two suckling mouse brain inocula, brain tissues harvested from young adult mice injected intracerebrally with either inoculum contained comparable titers of virus that was lethal for young adult mice and that produced only

small plaques. Further serial passages in young adult mouse brain continued to yield virus that was highly lethal for young adult mice (titers of $10^{8.0}$ to $10^{9.0}$ $MICLD_{50}$/ml) and that produced only small plaques (titers of $10^{6.8}$ to $10^{8.0}$ pfu/ml).

Discussion

One objective of this study was to compare virulence and plaque size properties of an attenuated (20t) strain of VEE virus with those of the small plaque-forming, moderately attenuated 9t strain and the large plaque-forming, virulent PES strain. The 20t strain was derived from the 9t strain (1), which had been obtained from L cell cultures inoculated with the PES strain (2). An understanding of the behavior of these strains was of value to us because of their use in other studies designed to elucidate mechanisms that may be involved in the attenuation of VEE virus and perhaps other viruses in vitro.

In a previous study, we attempted to recognize and describe some of the events that occurred during the attenuation of our virulent strain of VEE virus during passage in vitro (5). That attenuation of the virus was closely associated with diminished plaque size was clearly evident. Similar results have been obtained by other investigators with VEE and other viruses (6). Moreover, we have taken advantage of this information in developing techniques for the selection and passage of small plaque virus in order to derive fresh, attenuated strains to replace depleted or undesirable stocks that had lost titer through storage. One such passage attempt led to the isolation of the highly attenuated 20t strain (1).

We have found previously, however, that diminished plaque size and attenuation are not irrevocably linked to one another (7). A few passages of the attenuated 9t strain in mice produced a strain that had retained its small plaque characteristic but lost its attenuation for these animals. Like the virulent PES virus, the new strain was lethal by the intraperitoneal route, but unlike the PES strain it failed to multiply to any extent in spleen tissue (7). Moreover, any doubt as to the dissociation between plaque size and virulence of VEE virus per se was dispelled by the work of Zarate and Scherer (8), who examined a large number of isolates without finding a uniform correlation between these two properties.

The present work has resulted in the isolation of a large plaque attenuated strain and the isolation again of a small plaque virulent strain. Based upon these and previously reported results, we tentatively hypothesize that, while plaque size and virulence are unrelated for VEE virus per se, strains showing a definite relationship between these two properties can be obtained by deliberate passage in specific host systems. Thus, passage of the large plaque virulent PES strain in L cells produced small plaque attenuated virus, passage in mice yielded small plaque virulent virus, and passage through L cells and then in embryonated eggs was conducive to large plaque attenuated virus selection. Passage in embryonated eggs without prior passage in L cells continued to yield large plaque virulent virus (9).

Notwithstanding the apparent stability of plaque and virulence properties for a virus continuously passed in a suitable host, it is clear that a change in host or specific tissues within a host could result in significant alteration of biologic properties. Within the viral populations obtained during many of these passages, a number of intermediate types can be found (1, 5, 7, 9). These viral forms appear to be complex and unstable. There is evidence that they may yield to selective environmental pressures and change into forms possessing relatively stable virulence and plaque-forming characteristics, but they are not well understood.

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